

# THE METABOLIC CHARACTERISTICS OF NUCLEOLAR, CHROMOSOMAL, AND CYTOPLASMIC RIBONUCLEIC ACID OF *DROSOPHILA* SALIVARY GLANDS

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## ABSTRACT

Incorporation and retention of adenine-8-C<sup>14</sup> and of P<sup>32</sup>O<sub>4</sub> by nucleolar, chromosomal, and cytoplasmic RNA have been studied. Radioisotope concentrations were determined from autoradiographs, by grain counting, and RNA concentrations by microphotometry after basic staining. The relation between rates of RNA accumulation and rates of adenine incorporation was used to determine if synthesis was used to replace RNA which was lost from a fraction, and to obtain estimates of turnover rate. Nucleolar incorporation patterns indicate its incorporation is independent of growth, and there is complete turnover of the fraction in an hour or less. Nucleolar turnover is attributed to degradation of RNA within the nucleolus rather than to movement of intact molecules from the nucleolus. Chromosomal RNA reaches a much lower maximum specific activity than nucleolar, and a slightly higher maximum than cytoplasmic RNA. It showed faster incorporation than cytoplasmic RNA while accumulating RNA at the same rate as the cytoplasm, suggesting chromosomal RNA turnover. No evidence of cytoplasmic RNA turnover was found: rate of incorporation and rate of growth were correlated, and retention studies detected no decrease in amount of RNA-C<sup>14</sup>, RNA-P<sup>32</sup>, or RNA. Different ultimate precursors are indicated for nucleolar and non-nucleolar RNA by the observation that the nucleolar precursor is labeled before the precursor of non-nucleolar RNA.

Tracer studies have shown that RNA synthesis is a common phenomenon in a wide variety of tissues (1). Incorporation by tissues which are not growing indicates that newly synthesized RNA is needed to replace RNA which is continually lost; but it has not been clear whether RNA is lost from cellular structures during function, or is degraded in processes completely unrelated to function (2).

The salivary gland of *Drosophila* is ideal material, in several respects, for studying RNA. A major advantage is the absence of cell death, cell division, and mitosis—special events expected to involve losses and syntheses of RNA, but not to be confused with the usual behavior of RNA during its function in “resting” cells. In addition, microphotometric and autoradiographic measur-

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ing problems are minimal with salivaries, since very large cellular structures result from growth without cell division. The small size of nucleoli and nuclei in the usual cell makes it difficult to measure nucleoli without chromatin, and chromatin without nucleoli. But photometry of salivary structures is relatively simple; and emitters of fairly hard radiation, more reliable for quantitative autoradiography than the very soft radiation needed for high resolution, can be localized in terms of nucleoli, chromatin, and cytoplasm.

Earlier work with salivaries of *D. repleta* showed that nucleolar incorporation of  $P^{32}O_4$  is very fast, chromosomal incorporation fairly slow, and cytoplasmic incorporation slowest (3). The significance of incorporation rates in terms of functional characteristics of the different RNA fractions has been further examined in the investigations reported here. The studies have attempted to distinguish replacement synthesis from growth synthesis, to estimate rates of replacement synthesis, and to demonstrate directly, by retention studies, losses of labeled RNA from structures within the cell.

To accomplish these ends, the incorporation of adenine-8- $C^{14}$  by nucleolar, chromosomal, and cytoplasmic RNA have been compared, the relation between rates of incorporation and rates of RNA growth determined, retention of adenine- $C^{14}$  and  $P^{32}$  followed over 2 day periods, and incorporation patterns shown with  $P^{32}$  and adenine- $C^{14}$  compared.

## MATERIALS AND METHODS

Larvae were taken from a fairly uniform culture at the desired stage, and labeled precursor administered to them in their food for 2 hours, after which they were fed food without isotope. Adenine-8- $C^{14}$  was used at a concentration of 5  $\mu$ c. per cc. of food (specific activity of 3.7 mc./mmole, Schwartz), and  $P^{32}$  at 150  $\mu$ c. per cc. The same preparation of food with adenine- $C^{14}$  and the same culture of larvae served for the two adenine experiments reported.

Food and growth conditions for larvae, and experimental procedures followed have been described in detail previously (3-5). Larvae were fixed by freeze-substitution with post treatment in hot alcohol, cross-sections cut at 4  $\mu$ , and only the most distal cell layers of the gland studied. All slides were treated with cold trichloroacetic acid to remove acid soluble materials before applying film.

$P^{32}$  slides were exposed for 14 days. Several different exposures were used for adenine: 24 days in the first experiment; in the second experiment, one series of

slides was exposed 14 days, and a second one exposed 42 days to permit more accurate counting of low activities in chromatin and cytoplasm; all adenine- $C^{14}$  data are expressed here in terms of a 24-day exposure. (Cytoplasmic counts of the same larvae, made after different exposure times, indicated grain count per unit area was proportional to exposure time.)

Grains were counted in 40  $\mu^2$  areas of film above nucleoli, and in 40 or 160  $\mu^2$  areas above chromatin and cytoplasm. (The larger area was used when counts were low, to increase accuracy.) A slide with no nucleic acid extracted and a slide treated with ribonuclease were counted, and RNA activity determined as the difference between the two. (Nucleolar and cytoplasmic nucleic acid- $C^{14}$  was completely removed by ribonuclease.) Background counts were made for each slide, and values corrected accordingly.

Grain densities which were measured throughout all experiments were within the range in which grain count per unit area is directly proportional to exposure. Grain count per unit area, therefore, reflects the amount of label (associated with RNA) in a unit volume of tissue and is referred to as relative RNA- $C^{14}$  or RNA- $P^{32}$  concentration. Specific activity is the amount of label in a unit amount of RNA, and was calculated by dividing grain count per unit area by RNA concentration.

Sections adjacent to those used for autoradiographs were stained with the basic dye azure B bromide (1 mg./cc., pH 4, for 2 hours at 40°C., followed by tertiary-butyl alcohol overnight), and the concentration of stain in nucleoli and cytoplasm measured and taken as estimate of their RNA concentration. Extinctions at 480 m $\mu$  were measured with a microspectrophotometer, using small areas of 5 or more cells of a larva. Means of 3 larvae were averaged for most samples, of 2 in some. Chromosomal RNA is not stained under the conditions used here, but a different method of estimate used in a previous study indicated its concentration was about 37 per cent of the concentration found in nucleoli and cytoplasm (which are about the same) during 2nd day of 3rd instar (3). 37 per cent of the average of the nucleolar and cytoplasmic extinctions was used as the chromosomal value for the 2nd day and so long as there were no marked changes in cytoplasmic or nucleolar RNA concentrations.

Volumes of nuclei and nucleoli were calculated from micrometer measurements of their diameters. The ratio of cytoplasmic to nuclear volume was determined from camera lucida drawings of serial sections, by planimetry, or by weighing cut-outs of nuclei and cytoplasm (from traces made on glassine paper). The nuclear volume, determined from diameter measurements, was multiplied by this ratio to calculate cytoplasmic volume.

All data on volumes, basophilia, grain counts, etc.

have been calculated by averaging means from individual larvae; standard errors, therefore, indicate variations among larvae of a sample. Variations among cells of an individual larva were usually small, and a sample of from 5 to 10 cells was sufficient to determine the value for a single animal. Volumes and grain counts were determined from samples of 3 or more animals; for cytoplasmic volumes only 1 or 2 larvae were studied for a given sample.

#### OBSERVATIONS AND RESULTS

Data from a few larvae are shown in Fig. 1 to demonstrate the general changes in nucleolar and cytoplasmic RNA which occur during the first 4 days of 3rd instar. The first days can be described as a growth phase, with structures increasing in size and RNA content. Nucleolar growth is limited to the first 2 days, with a maximum size and RNA content being reached on the 3rd day,

after which both decrease. On the 3rd day cytoplasmic changes are also seen: there is a decrease in RNA concentration, and mucoprotein secretory granules (6, 7) appear and accumulate there. This 3rd day can be characterized as a period of differentiation, since there are changes in the cell's morphology, chemical composition, and relative sizes of the cellular structures.

Retention experiments were designed to cover the period of differentiation, since the observed decreases in RNA concentration and amount during this period—as well as general considerations—implicated this period as the one most likely to reveal loss of RNA.

Incorporation of adenine was studied in two different experiments. It was administered to early 2nd day larvae in the first experiment, and relative growth rates of the three RNA fractions

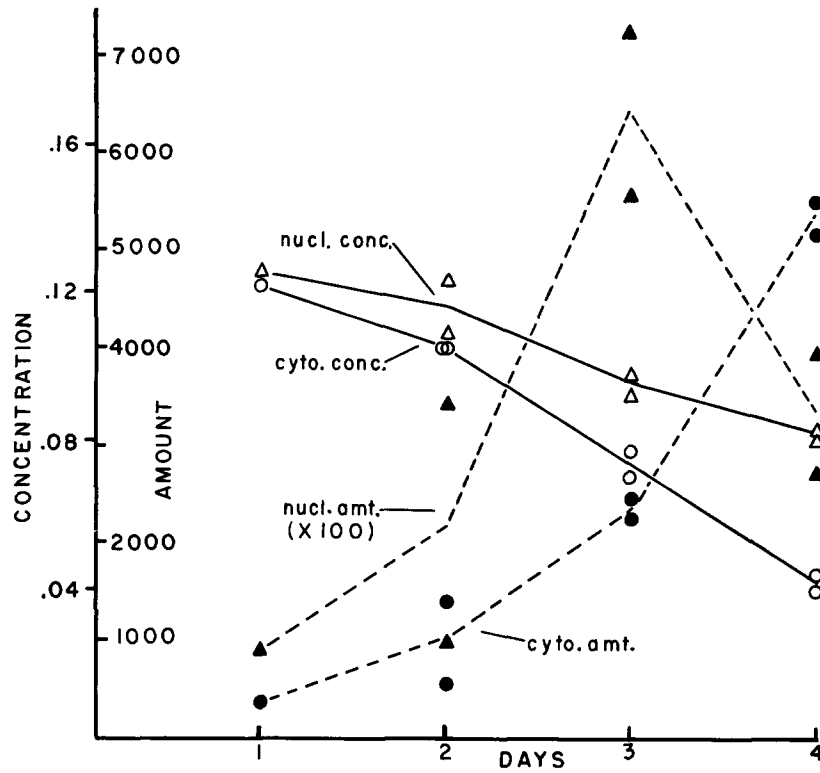


FIGURE 1

Concentrations and amounts of RNA in distal cells of salivary gland on different days of 3rd instar. From a culture not used in labeling experiments. Each point represents the average value for about 10 cells of a single larva.  $E_{480\text{m}\mu}/\mu$  is the unit of concentration.  $E_{480}/\mu$  times volume (in cubic micra) is the unit of amount. Nucleolar amounts were multiplied by 100 for easier plotting.

used to determine the significance of relative rates of incorporation. In the second experiment, adenine was administered 19 hours later when nucleoli were not growing and other fractions were growing more slowly than in the first experiment; comparison of the two experiments was used to determine if incorporation rate of a fraction is correlated with its growth rate.

Details of incorporation were studied after adenine administration on 2nd day. Figs. 2 through 7 demonstrate the material.

The first point of interest is a detectably earlier onset of incorporation by nucleoli than by the other fractions. Table I shows counts for individual larvae after an hour's feeding on adenine-C<sup>14</sup>. All larvae (and all nucleoli of each larva) have high nucleolar counts; in 2 larvae neither cytoplasmic nor chromosomal counts differed from background; the 3rd larva had low activity in chromatin but none in cytoplasm. (A cursory study of adenine incorporation by 1st day larvae, not given in detail here, gave similar data. Counts (averages of 2 larvae) at 1 hour were: nucleolus 43.6, cytoplasm 2.2, chromatin 1.3, background 1.2; and at 2 hours, 52.8, 3.2, 7.2, and 3.4.)

As indicated in Fig. 8, the nucleolus reaches a much higher maximum concentration of RNA-C<sup>14</sup> than the other fractions. The chromatin reaches a low maximum, but its rate of increase is very fast; it begins incorporating about an hour after ad-

TABLE I  
Grain Counts (per 160 μ<sup>2</sup>) after 1¼ Hours' Feeding on Adenine-8-C<sup>14</sup>

Background count of 3.7 not subtracted.			
Larva	Nucleolus	Cytoplasm	Chromatin
1	71.2 ± 7.2	1.5 ± 0.4	4.6 ± 2.2
2	110.0 ± 16.8	3.4 ± 0.3	11.4 ± 3.7
3	72.8 ± 8.7	0.6 ± 0.9	1.7 ± 0.8

ministration, and the maximum is reached by the 2nd hour. The cytoplasm shows the slowest incorporation, approaching a maximum at about 4 hours.

The amounts of C<sup>14</sup> incorporated per unit of RNA were calculated from RNA concentrations, and are shown in Table II. Nucleolar RNA is much more extensively labeled by adenine-C<sup>14</sup> than either of the other fractions. Although the maximum concentration of RNA-C<sup>14</sup> found in the chromatin was low, the chromosomal RNA actually reaches a higher specific activity than the cytoplasmic RNA.

The changes in volumes and RNA content during the course of the experiment, shown in Figs. 9 and 10, demonstrate in some detail the growth patterns during 2nd and 3rd day. Structures increased in size at a fairly constant rate during the 2nd day; abrupt changes in growth patterns of all fractions are seen on the 3rd day.

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Microphotographs of 4 μ cross-sections of salivaries. Larvae from first adenine experiment. Figures on left photographed with phase microscope, focused on tissue; figures on right made with bright field, focused on grains above tissue. Magnification in Figs. 2 and 3 approximately 1700, in others approximately 1100.

FIGURES 2 AND 3

From a larva fixed 1¼ hours after it began eating adenine-C<sup>14</sup>, showing fairly high grain count above nucleolus in the upper cell. Radiation always diverges from a labeled nucleolus into the film above the perinucleolar area, which contains chromatin. To determine chromosomal activity with error due to scatter from the nucleolus eliminated, counts were made above nuclear sections with no nucleolar material included, such as in the lower cell. There are very few grains above this nuclear section, or above the cytoplasm.

FIGURES 4 AND 5

From a larva fixed shortly after a 2 hour feeding on adenine-C<sup>14</sup>. Note grains above chromatin and cytoplasm, and high nucleolar count.

FIGURES 6 AND 7

From a larva fixed 17 hours after a 2 hour feeding on adenine-C<sup>14</sup>. Similar grain counts above all structures.

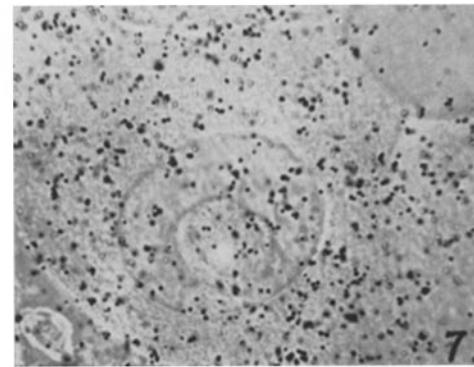
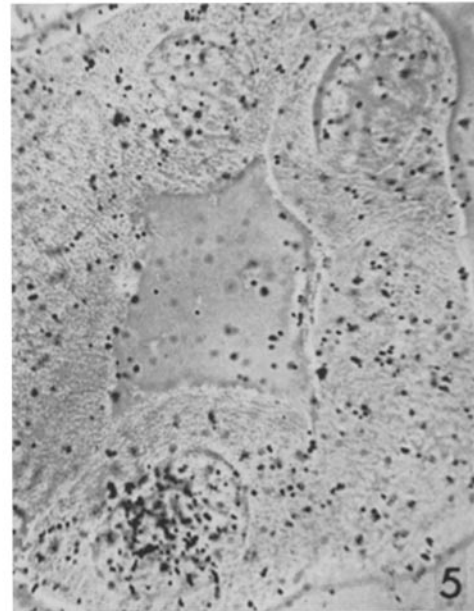
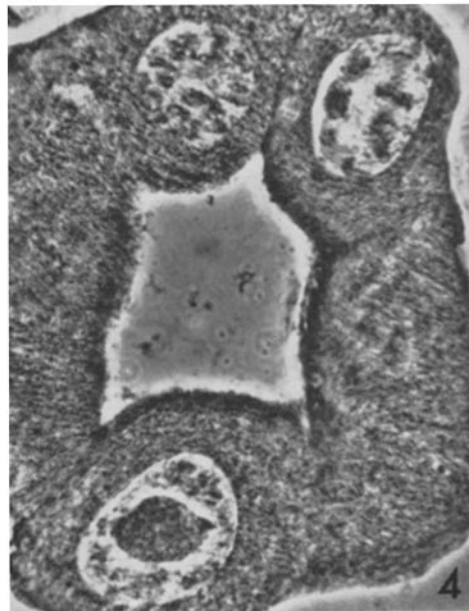
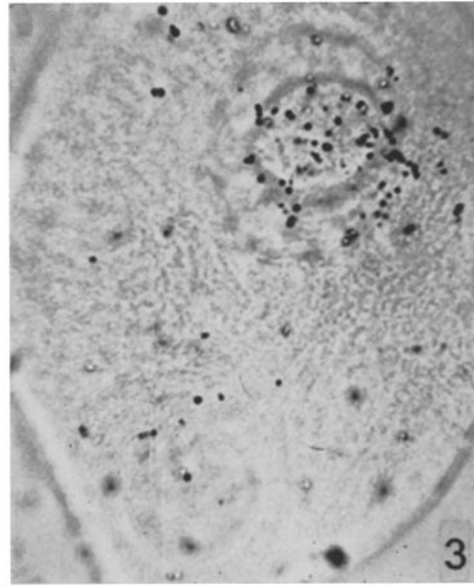
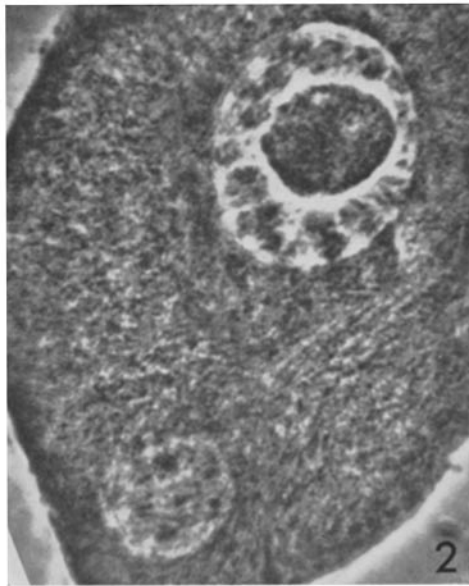


TABLE II

*Adenine-8-C<sup>14</sup> Administered on 2nd Day of 3rd Instar*

Grain counts per 160  $\mu^2$  (with standard errors) indicate RNA-C<sup>14</sup> concentrations. E<sub>480</sub> (extinction at 480 m $\mu$  of 4  $\mu$  sections stained with azure B) indicates RNA concentration; E<sub>480</sub> values in parentheses were not measured directly, but were estimated from succeeding samples. Calculation of relative specific activities explained in text.

Time	Nucleolus			Cytoplasm			Chromatin	
	Grain count	E <sub>480</sub>	Specific activity	Grain count	E <sub>480</sub>	Specific activity	Grain count	Specific activity
<i>hrs.</i>								
1 $\frac{1}{4}$	79.1 ( $\pm 10.0$ )	(0.359)	220	0.4 ( $\pm 0.8$ )		0	2.4 ( $\pm 3.1$ )	18
2 $\frac{1}{4}$	177.8 ( $\pm 11.9$ )	(0.359)	495	21.5 ( $\pm 2.5$ )	(0.351)	61	24.8 ( $\pm 2.5$ )	188
4 $\frac{1}{4}$	121.0 ( $\pm 8.1$ )	0.365	331	45.9 ( $\pm 3.2$ )	0.349	132	25.9 ( $\pm 5.2$ )	196
8	73.6 ( $\pm 10.8$ )	0.356	207	48.5 ( $\pm 6.7$ )	0.353	138	23.3 ( $\pm 7.1$ )	173
19 $\frac{1}{2}$	29.4 ( $\pm 4.0$ )	0.387	76	50.6 ( $\pm 5.4$ )	0.313	162	19.0 ( $\pm 5.7$ )	147
26	20.9 ( $\pm 0.8$ )	0.347	60	43.4 ( $\pm 4.8$ )	0.311	140	18.6 ( $\pm 3.4$ )	153
32	17.8 ( $\pm 3.3$ )	0.343	52	30.7 ( $\pm 2.6$ )	0.214	144	10.7 ( $\pm 3.6$ )	—
47 $\frac{1}{2}$	14.8 ( $\pm 3.3$ )	0.268	55	16.6 ( $\pm 2.2$ )	0.117	142	3.0 ( $\pm 5.2$ )	—

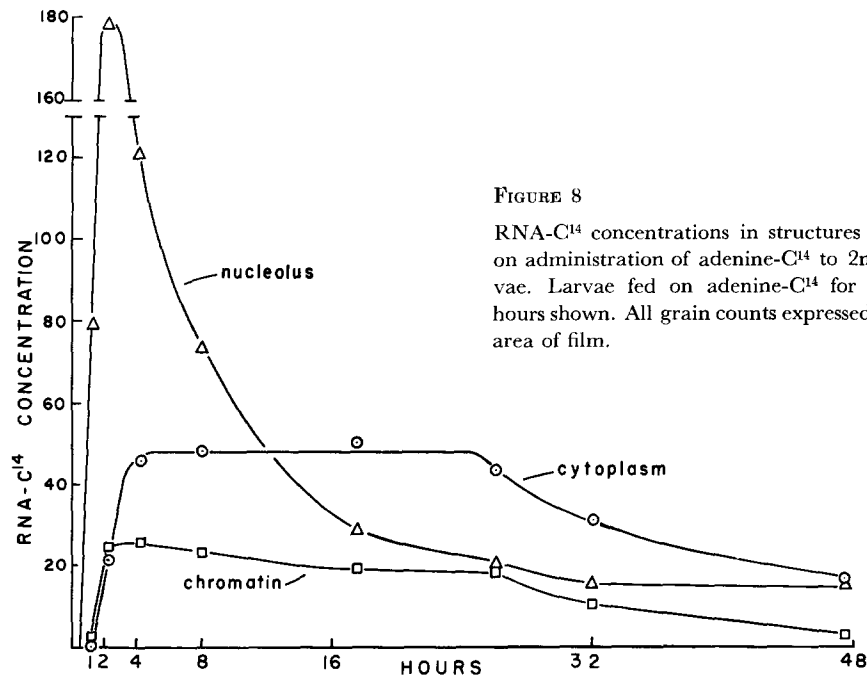


FIGURE 8

RNA-C<sup>14</sup> concentrations in structures of the cell on administration of adenine-C<sup>14</sup> to 2nd day larvae. Larvae fed on adenine-C<sup>14</sup> for the first 2 hours shown. All grain counts expressed for 160  $\mu^2$  area of film.

The percentage by which RNA was increasing at the time adenine- $C^{14}$  was administered was calculated from these curves and indicated similar growth rates for all RNA fractions. Each fraction increased in amount by 10 to 12 per cent during the initial hour of the experiment.

In their 1st hour of synthesis of labeled RNA, the chromosomal RNA and the cytoplasmic RNA reach specific activities in the ratio of 3 to 1, as seen in Table II, which indicates the rate of synthesis of chromosomal RNA is thrice that of cytoplasmic RNA, if the fractions derive their  $C^{14}$  from the same source. If chromosomal RNA is synthesized at a higher rate than cytoplasmic RNA, yet accumulates at about the same rate, the extra synthesis must be used to replace RNA which is lost, and chromosomal turnover is indicated. If cytoplasmic loss occurs, its rate must be lower than the chromosomal.

Nucleolar RNA obviously incorporates more  $C^{14}$  per hour than the other fractions, indicating more synthesis and a higher rate of replacement if it is assumed nucleolar RNA has the same precursor as the other fractions. This precursor situation seems unlikely; the 1 hour counts and the origins of the curves indicate no incorporation by cytoplasm and chromatin for a significant time when the nucleolus is incorporating. Nucleolar incorporation of a labeled substance while "non-nucleolar" RNA is incorporating substances not yet labeled clearly implies non-identity of their respective precursors. (Only two points determine the chromosomal origin, Fig. 8, and interpretation rests heavily on the 1 hour counts. There is no reason to think experimental error lowered these particular counts, especially since non-nucleolar RNA- $C^{14}$  was also absent at 1 hour in 1st day larvae, and  $P^{32}$  curves show late cytoplasmic and

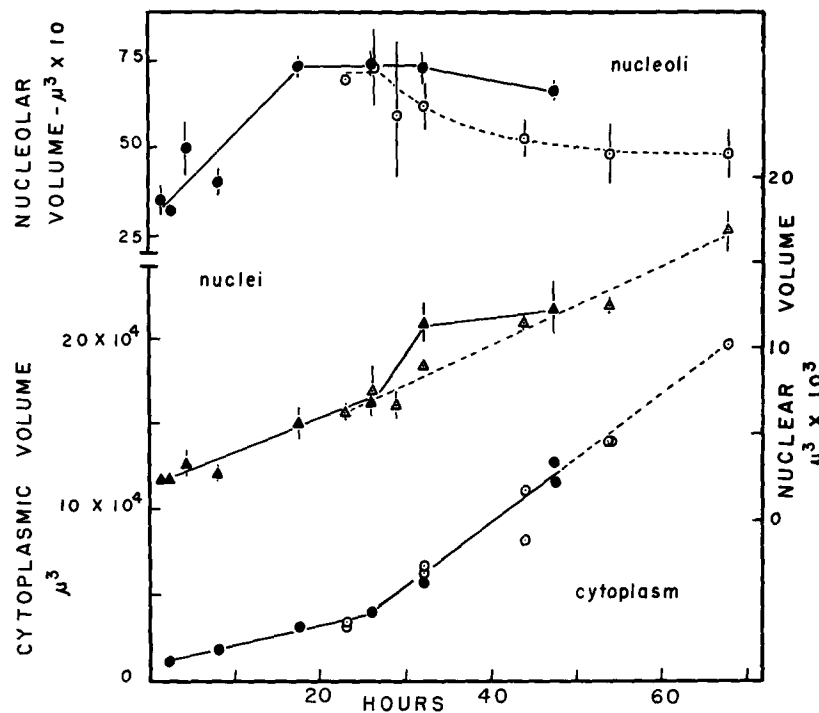


FIGURE 9

Volumes of structures in cells of the first adenine experiment (solid symbols) and of the  $P^{32}$  experiment (open symbols). Cytoplasmic points represent single larvae; nuclear and nucleolar data represent averages, and standard errors, of several larvae in each sample. The 3 day period plotted begins with the 2nd day of 3rd instar at the time adenine- $C^{14}$  was administered. Data from the two experiments were plotted on the same time scale by superimposing times at which secretory granules first appeared.

TABLE III

Administration of Adenine- $C^{14}$  to Larvae on 3rd Day of 3rd Instar

Concentrations of RNA- $C^{14}$  and RNA are indicated by grain counts and  $E_{480}$ . Also shown are average nucleolar volumes with standard errors, and estimated relative specific activities of chromosomal RNA.

Time	Nucleolus			Cytoplasm		Chromatin	
	Grain count (per 160 $\mu^2$ )	$E_{480}$	Volume $\mu^3$	Grain count (per 160 $\mu^2$ )	$E_{480}$	Grain count (per 160 $\mu^2$ )	Specific activity
hrs. 3	209 ( $\pm 8.9$ )	0.395	851 ( $\pm 59$ )	20.5 ( $\pm 1.3$ )	0.335	25.6 ( $\pm 4.3$ )	190
6	91.5 ( $\pm 17.1$ )		1083 ( $\pm 125$ )	29.4 ( $\pm 5.0$ )		11.6 ( $\pm 5.0$ )	83
9	67.3 ( $\pm 7.7$ )		851 ( $\pm 31$ )	38.5 ( $\pm 4.5$ )		12.1 ( $\pm 4.7$ )	87
12	60.0 ( $\pm 0.6$ )	0.418	914 ( $\pm 148$ )	50.3 ( $\pm 5.2$ )	0.357	10.0 ( $\pm 5.2$ )	72
28	26.0 ( $\pm 5.7$ )	0.365	677 ( $\pm 50$ )	32.4 ( $\pm 2.5$ )	0.203	12.8 ( $\pm 4.6$ )	92

chromosomal origins; straight line curves found with long  $P^{32}$  administration (3) are clearer on this point than the  $P^{32}$  curves below.)

To distinguish between growth synthesis and replacement synthesis of nucleolar RNA, incor-

poration by growing and by non-growing nucleoli were compared (Fig. 11). Nucleolar RNA was increasing in 2nd day larvae, while 3rd day nucleoli were at their maximum size and RNA (Fig. 10 and Table III). The correspondence

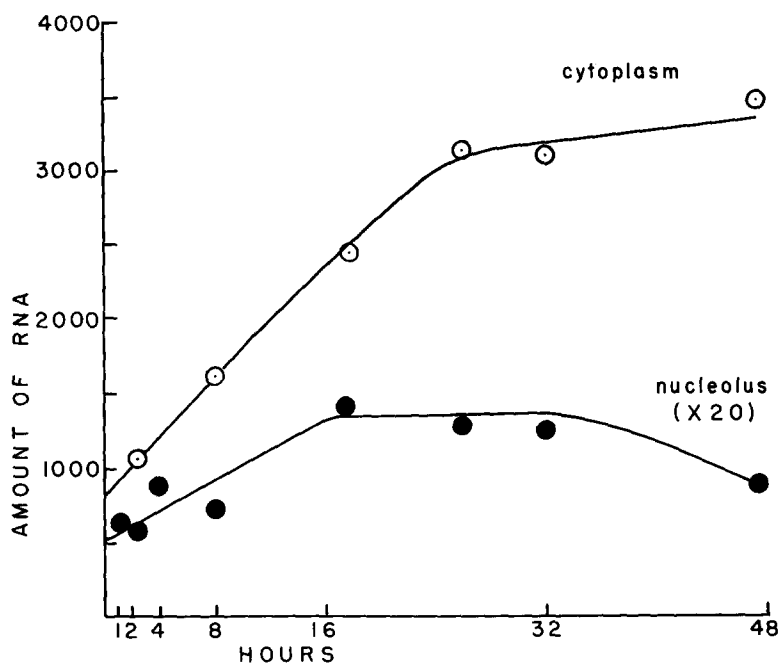


FIGURE 10

Amounts of nucleolar and cytoplasmic RNA during the 2nd and 3rd days of 3rd instar. Calculated as  $E_{480}$  times volume (in  $\mu^3$ ) from average values found during the course of the first adenine experiment.



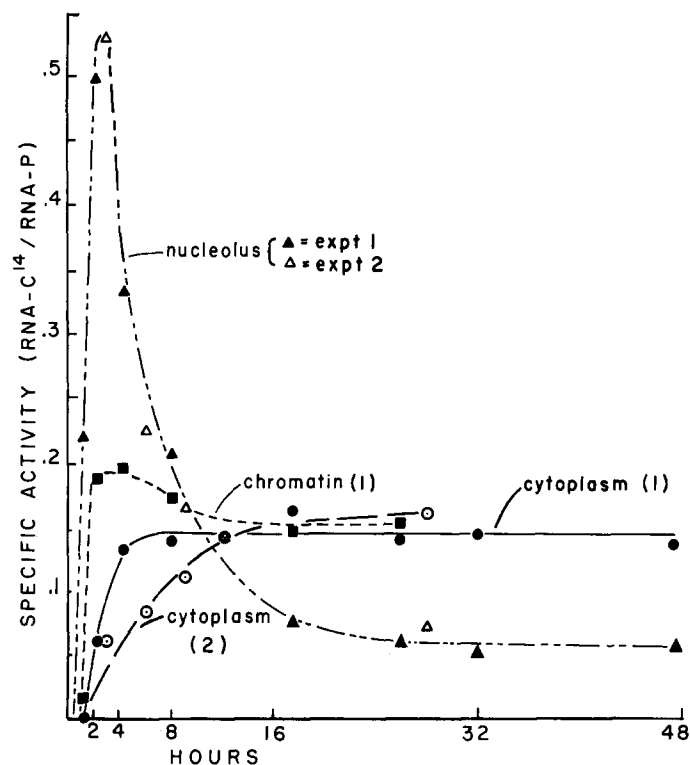


FIGURE 11

Comparison of relative specific activities of RNA fractions after adenine- $C^{14}$  administration to 2nd day larvae (first experiment) and to larvae a day older (second experiment.)  $C^{14}$  per unit RNA was calculated as grains per  $160 \mu^2$  divided by  $E_{48c}$ .

between incorporation curves is striking, and indicates that the nucleolar incorporation reflects replacement synthesis. Since growth synthesis, occurring at about 10 per cent per hour in the young larvae, did not perceptibly increase the total rate of incorporation, the rate of replacement synthesis must have been several times higher.

The growth rate of cytoplasmic RNA is also lowered in older larvae. As Fig. 10 shows, cytoplasmic RNA content increases (at the most) by a constant amount per unit time, therefore the percentage by which RNA increases becomes progressively lower. Older larvae show a slower increase to maximum specific activity than the young larvae, as seen in Fig. 11, indicating incorporation rate of cytoplasmic RNA is dependent on its growth rate.

Chromosomal RNA reached about the same maximum specific activity, and at about the same time, in the two experiments. (See Tables II and III.) Presumably, there was a lower growth

rate in the older larvae, but incorporation rates indicate no change in rate of synthesis.

The first adenine experiment was extended over a long period of time in order to study retention of labeled RNA.

As seen in Fig. 8, nucleolar RNA- $C^{14}$  concentration decreased almost immediately after adenine- $C^{14}$  administration was stopped. Fig. 11 shows that the  $C^{14}$  content per unit RNA decreases, and decreases to the same extent when the fraction is small and growing and when it is large and not growing. Hence, each unit of RNA undergoes the same loss and replacement of molecules in the two experiments. The  $C^{14}$  content per unit of RNA decreases by the same percentage in every unit of time, as shown by Fig. 12. The semilog plot of nucleolar specific activities (from the first experiment) indicates an exponential decrease to the minimum value found at the end of the experiment. (The minimum value was subtracted before plotting.) The specific activity at the beginning of

each hour decreased by about 20 per cent by the end of the hour.

The decrease in amount of RNA-C<sup>14</sup> per nucleolus (Fig. 13) is extensive and rapid, but, in growing nucleoli, less so than the decrease in specific activity. Specific activity and RNA-C<sup>14</sup> amount show parallel behavior in older nucleoli with a constant RNA amount, of course.

Chromosomal RNA showed only small decreases in specific activity in early hours after administration to young larvae, and no decrease in RNA-C<sup>14</sup> amount occurred until after the 32nd hour (Fig. 13). Cytoplasmic RNA-C<sup>14</sup> showed large decreases in concentration during granule accumulation (they were first apparent at the 32nd hour); equally large decreases in basophilia occurred at the same time (Table II), and no change in specific activity is indicated. Measurements of total amounts of RNA (Fig. 10) and of RNA-C<sup>14</sup> (Fig. 13) also failed to reveal loss of RNA from the cytoplasm.

P<sup>32</sup> was administered shortly before granule formation to follow retention during this period and subsequent hours. (Fig. 9 indicates the period studied.) Incorporation by 3rd day larvae shows no significant differences from 2nd day larvae (3) except for decreases in concentration of cytoplasmic and chromosomal RNA-P<sup>32</sup> after the 9th hour, seen in Fig. 14. Secretory granules first ap-

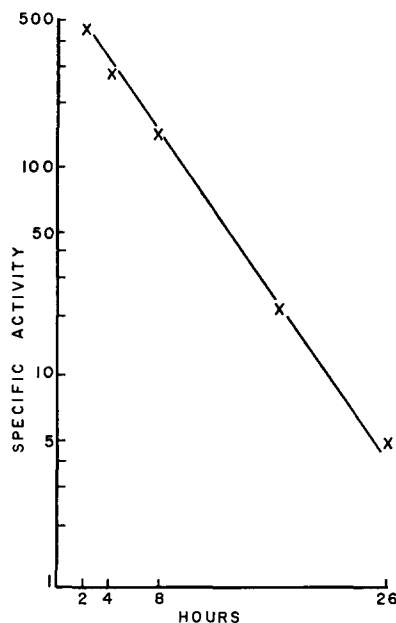


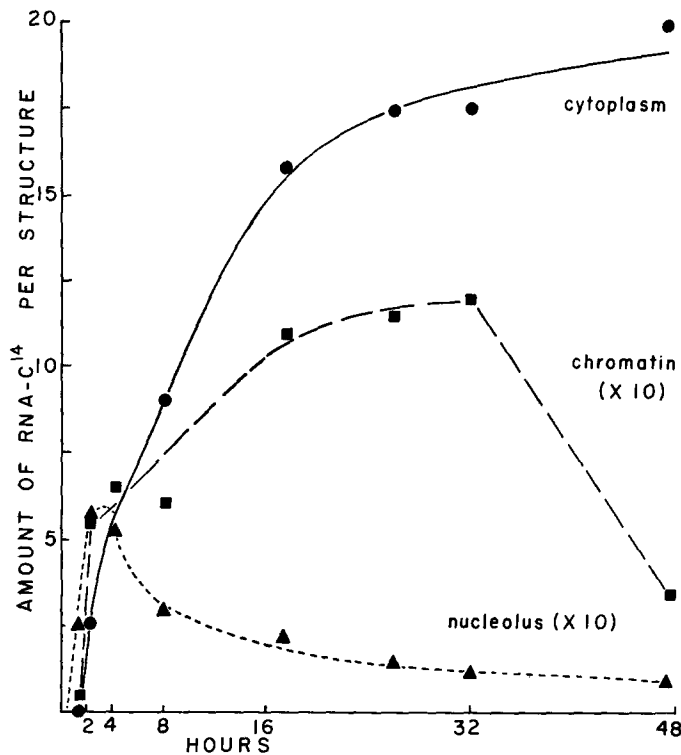
FIGURE 12  
Semilog plot showing exponential decrease in specific activity of nucleolar RNA to its minimum value. Data from first adenine experiment. The minimum value, 55, was subtracted before plotting.

peared in the cytoplasm at 12 hours, and measurements of basophilia before and after granule formation (Table IV) indicated cytoplasmic RNA

TABLE IV  
*P<sup>32</sup> Concentrations Present after Administration on 3rd Day of 3rd Instar*

Grain counts per 160  $\mu^2$  indicate total activity, found after extraction with cold TCA, and the activity which remained after extraction with RNase. Average counts and standard errors are given. Values in parentheses assumed from succeeding and/or preceding values. Also shown are azure binding of 6 and 34 hour samples.

Time	Nucleolus		Cytoplasm		Chromatin	
	Total	RNase-resistant	Total	RNase-resistant	Total	RNase-resistant
<i>hrs.</i>						
3	99.0 $\pm$ 5.1	(5.0)	13.3 $\pm$ 0.4	(1.5)	19.6 $\pm$ 2.0	(10.0)
6	102.0 $\pm$ 6.4	10.0 $\pm$ 2.4	43.6 $\pm$ 1.3	3.0 $\pm$ 1.0	42.6 $\pm$ 5.1	19.9 $\pm$ 0.7
9	78.0 $\pm$ 4.6	15.5 $\pm$ 0.8	51.4 $\pm$ 5.3	5.9 $\pm$ 2.9	55.6 $\pm$ 1.3	22.7 $\pm$ 2.1
12	68.2 $\pm$ 3.4	21.9 $\pm$ 1.2	51.8 $\pm$ 4.5	13.9 $\pm$ 5.4	60.6 $\pm$ 5.5	31.1 $\pm$ 2.2
24	54.3 $\pm$ 3.2	17.4 $\pm$ 4.2	57.9 $\pm$ 5.0	29.7 $\pm$ 10.2	55.9 $\pm$ 2.9	31.7 $\pm$ 2.1
34	57.3 $\pm$ 2.4	(19.7)	71.5 $\pm$ 2.2	41.4 $\pm$ 2.6	55.6 $\pm$ 3.2	32.0 $\pm$ 1.1
48			70.1 $\pm$ 5.6	45.2 $\pm$ 6.1	54.3 $\pm$ 8.9	32.7 $\pm$ 4.5
Time	Nucleolar E <sub>480</sub>		Cytoplasmic E <sub>480</sub>			
6	0.351		0.216			
34	0.346		0.141			



**FIGURE 13**  
Amounts of RNA-C<sup>14</sup> per structure after administration of adenine-C<sup>14</sup> to 2nd day larvae. Calculated as RNA-C<sup>14</sup> concentration (grains per 160 μ<sup>2</sup>) times volume (in μ<sup>3</sup>). Nucleolar and chromosomal values were multiplied by 10 for easier plotting.

concentration decreased, but its specific activity did not. (Since RNA concentration decreases gradually, the 12th hour specific activity was calculated from the mean of the two extinction measurements.) The nucleolus showed no change in RNA concentration, so its decreases in RNA-P<sup>32</sup> concentration represent decreases in specific activity. Chromosomal RNA concentration was not measured; the similarity in cytoplasmic and chromosomal curves of RNA-P<sup>32</sup> concentration suggests parallel changes in RNA of the two fractions.

Total amounts of P<sup>32</sup> in the various RNA fractions, Fig. 15, demonstrate loss of RNA from the nucleolus, but not from chromatin or cytoplasm. Decreases in RNA-P<sup>32</sup> concentration in cytoplasm and chromatin can be explained by absence of RNA accumulation while structures continue to enlarge during granule production. After this period both fractions again accumulate RNA-P<sup>32</sup> extensively. (Cytoplasmic RNA increase in 4th day larvae is indicated by Fig. 1.) Nucleoli have a pycnotic appearance in old larvae and are poor objects for quantitative study; they apparently do not increase in size or RNA content after the 3rd day.

#### DISCUSSION

The nucleolar RNA is of most interest here, since its behavior is seen with enough clarity to make some deductions about functional aspects of the fraction. The rapid incorporation and poor retention of adenine-C<sup>14</sup>, which it shows, are expected of a substance which is rapidly and continually metabolized. Its rate of incorporation is independent of growth of the fraction; that synthesis is needed to replace RNA which is being lost from the nucleolus is clear from the rapid decrease in specific activity and RNA-C<sup>14</sup> amount which occur.

The incorporation pattern observed reflects replacement synthesis, since the pattern is the same with no RNA growth and with growth at 10 per cent per hour. The kinetics of turnover can be determined from the observations, since the influence of growth is negligible, and indicate behavior of nucleolar RNA which is noteworthy for its simplicity.

The amount of RNA synthesized is directly proportional to the amount of RNA present, as shown by the comparison of adenine incorporation into large and small nucleolar fractions, and implies

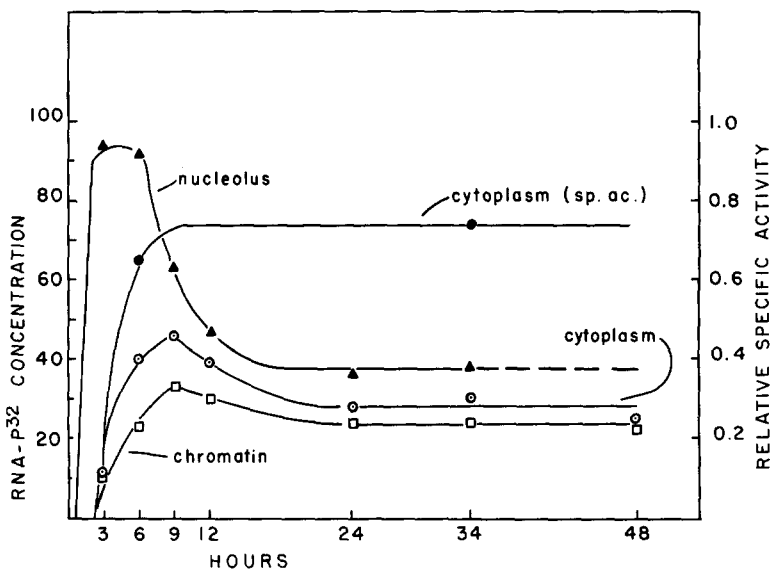


FIGURE 14

$P^{32}$  incorporation after administration to 3rd day larvae.  $P^{32}$  fed for the first 2 hours shown. Solid symbols indicate relative specific activity, read on the right scale, and open symbols indicate RNA- $P^{32}$  concentration (grains per  $160 \mu^2$ ), read on left scale; except for nucleoli, which had a constant RNA concentration, and the single curve is to be read on both scales. Specific activities obtained by dividing relative RNA concentration into grain count, with nucleolar concentration equal to 100, and cytoplasmic concentration equal to (cyto E/nucleolar E) times 100.

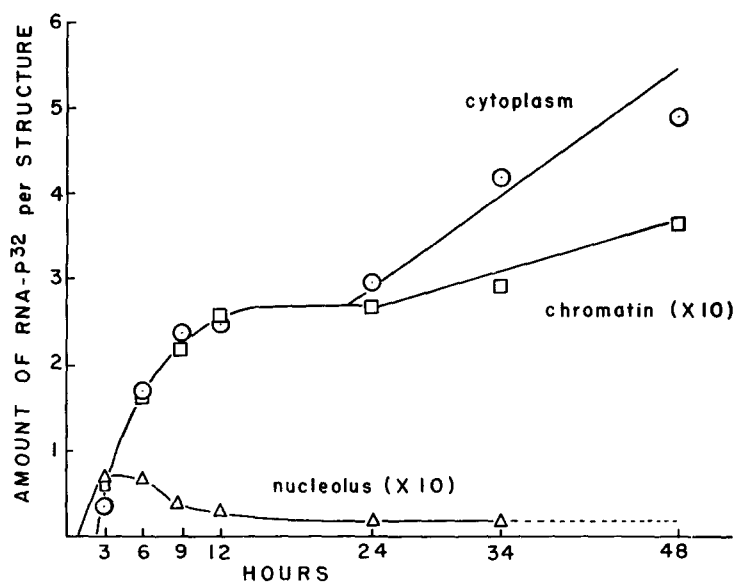


FIGURE 15

Amounts of RNA- $P^{32}$  per structure following administration to 3rd day larvae. Calculated as in Fig. 13. Chromosomal and nucleolar values were multiplied by 10 to facilitate plotting.

that a constant fraction of the total RNA is lost continually. Essentially instantaneous mixing of newly synthesized molecules with those already present, so that new molecules immediately begin disappearing (with the same frequency as old ones do), is indicated. The  $C^{14}$  content of RNA shows a large decrease by the 2nd hour after adenine- $C^{14}$  administration is stopped, and decreased to a smaller extent in each succeeding time interval, the decrease in each time interval being a constant proportion of the amount present during that time. (As shown by the exponential decrease to the minimum value, seen in Fig. 12.)

The early decrease in specific activity, the exponential curve, and the 20 per cent turnover per hour which it indicates, could occur under conditions of nucleolar turnover at this rate with synthesis from a precursor with essentially no activity (*i.e.*, a turnover rate much higher), or of nucleolar turnover at a much higher rate coupled with precursor turnover at 20 per cent. The latter condition is indicated to explain the essentially identical rates of synthesis in growing and non-growing nucleoli; for if RNA replacement synthesis occurred at only 20 per cent per hour, young nucleoli with synthesis sufficient for replacement plus growth at 10 per cent per hour should have shown a detectibly larger incorporation than nucleoli with only replacement synthesis (*i.e.*, in a ratio of 30 per cent to 20 per cent). A rate of nucleolar RNA turnover that accomplishes complete loss and replacement in an hour or less seems quite likely.

The nucleolar  $P^{32}$  curve does not show the early decrease in activity seen with adenine- $C^{14}$ ; but turnover is indicated by  $P^{32}$  incorporation without growth of RNA in the older larvae studied here, and a high rate is indicated, as with adenine- $C^{14}$ , by the sudden leveling off of activity when  $P^{32}$  is no longer fed. A very slow decrease in activity of the  $P^{32}$  precursor is, therefore, the probable explanation of the prolonged maintenance of maximum  $P^{32}$  activity.

The observations reported here are all consistent with the interpretation that nucleolar RNA is synthesized in the nucleolus and that it has a unique function which is accomplished there with degradation of the molecule. The rapid metabolism of nucleolar RNA strongly suggests that the RNA may be a precursor of other products which are useful in the cell; derivatives which are smaller than RNA are indicated as the products, as discussed below.

The "unitary" hypothesis of RNA (as aptly named by Swift (11)) postulates that RNA function is restricted to the cytoplasm, RNA synthesis is restricted to the chromatin, and the nucleolus is a temporary abode for RNA en route from chromatin to cytoplasm (10, 12). This hypothesis is based on the fact that high nuclear activity precedes high cytoplasmic activity, and assumes that the labeled RNA seen in the cytoplasm at some time after administration is the same labeled RNA that was in the nucleus at earlier times (8-10). In sum, any disappearance of labeled nuclear RNA, as found for the nucleolar fraction, is attributed to movement of intact molecules from the fraction, rather than to degradation of the molecules.

If nucleolar behavior accords with this scheme, we should expect nucleolar RNA molecules to reappear outside the nucleolus as "non-nucleolar" RNA when they disappear from the nucleolus. The evidence presented here that nucleolar RNA turns over at a very high rate indicates that labeled RNA will *begin* leaving the nucleolus essentially at the same time it begins appearing there. The "unitary" hypothesis is not supported, however, since labeled RNA does not begin to appear elsewhere in the cell until significantly later: the late appearance of label in "non-nucleolar" RNA is apparent with adenine- $C^{14}$ , and was clearly indicated by  $P^{32}$  studies reported previously (3). With  $P^{32}$ , cytoplasmic and chromosomal incorporation curves had origins at 2 hours after administration, while the nucleolar curve originated about an hour and a half earlier. A study of root tips also shows later onset of cytoplasmic cytidine- $H^3$  incorporation than of nucleolar incorporation (10).

Not only does labeled cytoplasmic RNA appear too late to have come from the nucleolus; label also appears in nucleolar RNA too early to have come from the chromosomal RNA. The *simultaneous* synthesis of labeled nucleolar RNA and of unlabeled non-nucleolar RNA is hard to explain except by assuming that the fractions are synthesized from different substances—nucleolar RNA from a substance which is labeled early (and loses label relatively fast), while cytoplasmic and chromosomal RNA require some other substance, or substances, which contain no label for some time (and lose label slowly).

The prolonged occurrence of precursor activity complicates interpretation of cytoplasmic and chromosomal retention data. Although decrease in amount of labeled cytoplasmic RNA was not

found with either adenine- $C^{14}$  or  $P^{32}$ , and decrease in amount of labeled chromosomal RNA was not found with  $P^{32}$ , turnover is not ruled out. In all these cases, the RNA must have had about the same specific activity as its precursor after the RNA maximum was reached, for there was no decrease in RNA specific activity while large amounts of newly synthesized RNA accumulated (Figs. 11, 13, 14, 15). Loss and replacement of RNA under these conditions would not have been detected.

Studies of other tissues have revealed cytoplasmic RNA loss which is difficult to explain by cell death and cell turnover (13, 14). Extensive turnover during the periods of salivary development followed here seems unlikely in view of the slow incorporation observed at all times, and the slower incorporation when growth was slower.

Adenine- $C^{14}$  reveals greater lability of chromosomal RNA than  $P^{32}$  does. Chromosomal RNA showed large losses of  $C^{14}$  during the 3rd day (discussed elsewhere (5)), and incorporated adenine much faster than the cytoplasm, without growing faster. The chromatin incorporates  $P^{32}$  only slightly faster than the cytoplasm ((3) and Fig. 14). More turnover of chromosomal RNA-adenine than of RNA-phosphorus is a possible explanation; another is slow turnover of the phosphorus precursor.

If we assume cytoplasmic and chromosomal RNA have the same  $C^{14}$  precursor and cytoplasmic turnover is negligible, the chromosomal incorporation rate (relative to the cytoplasmic) implies

chromosomal synthesis of 3 times as much RNA as needed to achieve growth at 10 per cent per hour, hence replacement synthesis at about 20 per cent per hour. This is much less turnover than indicated for nucleolar RNA, but is, nonetheless, extremely high (see discussion in (17)). Several autoradiographic demonstrations of incorporation by chromosomal or by nuclear RNA have been interpreted as indications of chromosomal turnover. Growing tissue has been used for most of these studies, however, and no data to indicate loss of chromosomal RNA, or to distinguish between growth and replacement synthesis, have been reported (10, 12, 18). Two studies have provided unambiguous evidence of turnover: chromosomal RNA incorporation in non-proliferating adult cells (rat pancreas (15) and salamander liver (16)) clearly demonstrates replacement synthesis.

A chromosomal maximum which is earlier than the nucleolar has been reported for root meristem (10) and tissue culture (12) as evidence that chromosomal RNA has a much shorter turnover time than nucleolar. This time-course of incorporation can hardly be considered a general rule in view of cases in which nucleolar RNA reaches a maximum before the chromatin (15, 3) or at the same time (found here). Relative speed with which these fractions reach maxima is, of course, unsuitable for judging their relative rates of synthesis if they have different precursors, as indicated in *Drosophila*.

#### REFERENCES

- SMELLIE, R. M. S., HUMPHREY, G. F., KAY, E. R. M., and DAVIDSON, J. N., *Biochem. J.*, 1955, **60**, 177.
- GRAHAM, A. F., and SIMINOVITCH, L., *Biochim. et Biophysica Acta*, 1957, **26**, 427.
- MCMASTER-KAYE, R., and TAYLOR, J. H., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 5.
- TAYLOR, J. H., and MCMASTER, R. D., *Chromosoma*, 1954, **6**, 489.
- MCMASTER-KAYE, R., and TAYLOR, J. H., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 461.
- LESHER, S. W., *Anat. Rec.*, 1952, **114**, 633.
- GAY, H., *Cold Spring Harbor Symp. Quant. Biol.*, 1956, **12**, 257.
- GOLDSTEIN, L., and MICOU, J., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 1.
- WOODS, P. S., and TAYLOR, J. H., *Lab. Inv.*, 1959, **8**, 309.
- WOODS, P. S., *Brookhaven Symp. Biol.*, 1959, **12**, 153.
- SWIFT, H., *Brookhaven Symp. Biol.*, 1959, **12**, 172.
- GOLDSTEIN, L., and MICOU, J., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 301.
- HECHT, L., and POTTER, V. R., *Cancer Research*, 1956, **16**, 988.
- HENDERSON, J. F., and LE PAGE, G. A., *Cancer Research*, 1959, **19**, 67.
- FITZGERALD, P. J., and VINIJCHAIKUL, K., *Lab. Inv.*, 1959, **8**, 319.
- SWIFT, H., Cytoplasmic particulates and basophilia, in Symposium on the Chemical Basis of Development, Baltimore, Johns Hopkins Press, 1958, 174.
- BENNETT, E. L., and KRUECKEL, B. J., *Biochim. et Biophysica Acta*, 1955, **17**, 503.
- ZALOKAR, M., *Nature*, 1959, **183**, 1330.